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An anti-phosphoinositide-specific monoclonal antibody that neutralizes HIV-1 infection of human monocyte-derived macrophages

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ABSTRACT

HIV-1 entry into cells requires the interaction of both HIV-1 envelope proteins and membrane lipids. We investigated the mechanism of neutralization of HIV-1 infection of primary monocyte-derived macrophages (MDM) by a murine monoclonal antibody (mAb) WR321. WR321 specifically binds phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. These phosphoinositides are present not only on the inner surface of the plasma membranes of cells but also on the surface of virions. HIV-1 acquires these lipids during the budding process. Pre-incubation of WR321 with the virus but not with MDM neutralized HIV-1 infection of MDM. Our results demonstrate that WR321 was internalized only when it was bound to HIV-1. WR321 did not prevent the entry of HIV-1 into MDM. However, once WR321 was internalized along with HIV-1 the mAb acted intracellulary to prevent the release of virions from MDM and also triggered the release of β-chemokines.

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Introduction

The search for an immunizing formulation that can induce broadly neutralizing antibodies against HIV-1 has proved to be challenging especially in view of the discovery that the broadly neutralizing human monoclonal antibodies (mAbs) 2F5 and 4E10, in addition to their specificity for protein epitopes in gp41 exhibit very broad lipid binding specificities. These mAbs bind to cardiolipin, phospholipids, cholesterol, glycosphingolipids, squalene, and lipid A (Haynes et al., 2005; Matyas et al., 2009b). There is strong evidence for the participation of the viral membrane in the neutralization process by the mAbs 2F5 and 4E10 (Alam et al., 2009) and recent studies show that binding of the antibodies to lipids could be part of the neutralizing mechanism (Haynes et al., 2005; Matyas et al., 2009b; Alving et al., 2006; Sanchez-Martinez et al., 2006). We have previously demonstrated the role and importance of lipids in binding and neutralization of HIV-1 with a panel of lipid-specific murine monoclonal antibodies. In those studies, murine IgM mAbs (WR301 and WR304) that exhibit only lipid binding activity and do not bind to recombinant gp120 or gp41 neutralized HIV-1 infection in the PBMC assay (Brown et al., 2007; Matyas et al., 2009b). The ability of anti-lipid antibodies to neutralize HIV-1 infection in the PBMC assay has also been demonstrated with several β2-glycoprotein 1-independent anti-phospholipid human IgG mAbs (PGN632, P1, IS-4, and CL1). (Moody et al., 2008, 2010). These observations are pertinent since it is well known that retroviruses including HIV-1 acquire their lipids during budding from the plasma membrane of a host cell (Nguyen and Hildreth, 2000).

In addition to WR301 and WR304, several other murine IgM and IgG mAbs have been generated in our laboratory and extensively characterized for their protein and lipid binding properties (Beck et al., 2008; Karasavvas et al., 2008). WR321 is a murine IgG mAb with exquisite specificity for phosphoinositides phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP2) (Matyas et al., 2010). Phosphoinositides that are recognized by WR321 do not exist on the external surface of cells. However, they are exclusively present on the cytoplasmic leaflet of the plasma membrane (Di Paolo and De Camilli, 2006). Plasma membrane associated PIP2 has been shown to be crucial for HIV-1 viral assembly, in particular for the assembly of Gag

Abbreviations: Ab, Antibody; GalCer, Galactosyl ceramide; HIV-1, Human immunodeficiency virus type 1; M-CSF, Macrophage colony stimulating factor; MPER, Membrane proximal external region; mAb, Monoclonal antibody; MDM, Monocyte-derived macrophages; PBMCs, Peripheral blood mononuclear cells; PI, Phosphatidylinositol; PIP, Phosphatidylinositol-4-phosphate; PIP2, Phosphatidylinositol-4,5-bisphosphate

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precursor protein p55 and any disruption in this reduces infectious virus production (Chan et al., 2008; Ono et al., 2004). In the present study, we have examined the mechanism of neutralization of HIV-1 by WR321 using a pure and defined subset of PBMCs, namely human monocyte-derived macrophages (MDM). WR321 was internalized after it bound to HIV-1. WR321 exerted its effect from within the cell by preventing the release of HIV-1 and by inducing the secretion of β chemokines.

Results

Characterization of in vitro generated human monocyte-derived macrophages (MDM)

Monocytes were isolated from PBMCs of HIV-1 seronegative donors using a cold agglutination procedure and cultured *in vitro* for 7 days. To ascertain that the *in vitro* differentiated cells were indeed MDM, cells were harvested and analyzed for the expression of various cell surface molecules by flow cytometry. As shown in Fig. 1, the cells were $CD14^+$, $CD11c^+$, $CD11b^+$, $CD3^-$, $CD20^-$, $CD40^-$, and $CD80^-$. The cells expressed high levels of class I (HLA-A, B, C) and class II (HLA-DR) molecules and low levels of CD4 and CD86 molecules on their cell surface. The cells expressed high levels of CD32 (FcγRII) and CD16 (FcγRIII), and were also positive for CD64 (FcγRI) and CD195. Phenotypic analyses showed that the cells were not T cells or B cells but expressed markers indicative of MDM.

In vitro infection of primary MDM with US-1 virus

MDM were infected with US-1 as described below. Cells were then washed and cultured for varying periods of time. Three different read-outs were utilized to determine the level of infection of MDM: (i) flow cytometry for the detection of intracellular HIV-1 p24 antigen in the infected MDM (Fig. 2A); (ii) ELISA for the quantitation of HIV-1 p24 antigen in the supernatants from the infected cultures (Fig. 2B) and (iii) electron microscopy for the intracellular detection of HIV-1 virions in infected MDM (Fig. 2C–E).

Following *in vitro* culture of infected MDM, there was a gradual increase in the percentage of MDM that were positive for HIV-1 p24 antigen by flow cytometry compared to uninfected MDM (Fig. 2A). A representative experiment with the percentage of infected cells in each of the panels is shown. At day 3 post-infection, the percentage (mean ± S.D.) of infected MDM from three independent experiments was 5.60 ± 0.91%. This number increased significantly over time. The percentage of infected MDM was 14.30 ± 3.78% ($p=0.01$), 27.97 ± 7.12% ($p=0.005$), and 44.03 ± 4.58% ($p=0.0001$), respectively on days 5, 7, and 9 post-infection. There was a concomitant increase in the amount of p24 present in the culture supernatants of these cells (Fig. 2B). The concentration of HIV-1 p24 antigen in the corresponding culture supernatants increased significantly ($p \leq 0.04$) on days 3, 5, 7, and 9 post-culture compared to uninfected cultures (Fig. 2B). Numerous mature HIV-1 virions and virions at various stages of budding were detected inside intracytoplasmic vacuoles of HIV-1-infected MDM on day 10 post-infection (Fig. 2C–E) indicating a productive infection.

Neutralization of HIV-1 infection of MDM by mAbs

The lipid specificities of WR321 are shown in Table 1. In the initial experiments, MDM differentiated from a single HIV-1 seronegative donor were used to evaluate the ability of WR321 to neutralize US-1 virus infection. The human IgG mAbs, 2F5 (specific for both HIV-1 envelope protein and lipids), and CL-1 (specific for lipids), were used as control mAbs.

Two separate neutralization assay formats, preincubation of WR321 with MDM (cell format) and preincubation of WR321 with HIV-1 (virus format) were utilized. In the cell format (Fig. 3), MDM were preincubated with WR321 before infection with purified US-1 virus as described in the Materials and methods section. The mAb were tested in triplicate at a final concentration of 10 μg/ml and neutralization of the virus above 50% was considered positive. The human IgG mAbs, 2F5 and CL1, were

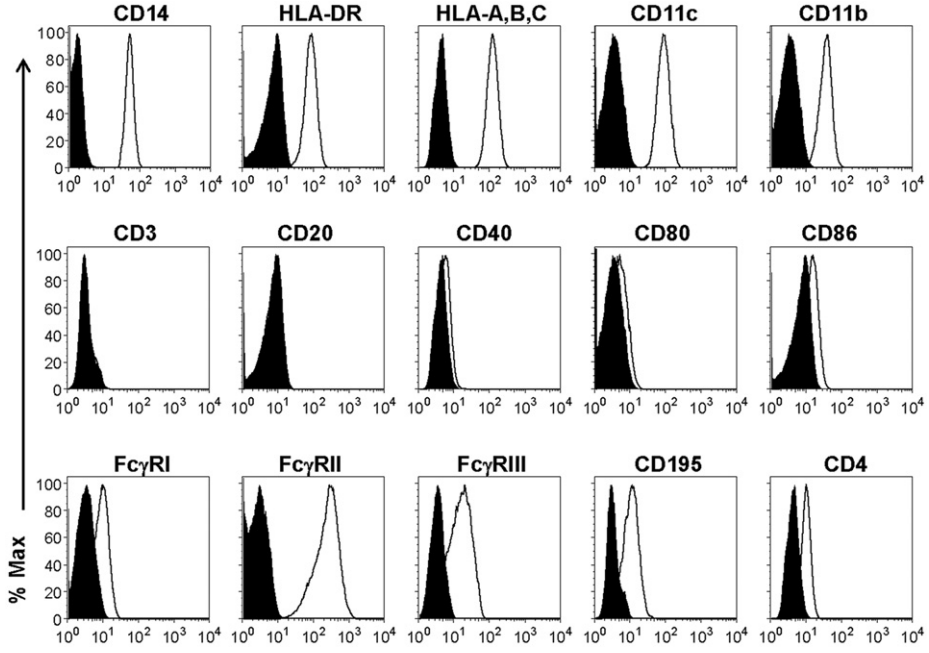

Fig. 1. Phenotypic analyses of MDM. Monocytes were isolated from PBMCs of an HIV-1 seronegative donor and differentiated into MDM following *in vitro* culture. The cells were harvested on day 7 post *in vitro* culture, stained with a cocktail of mAbs, and analyzed by flow cytometry for the expression of cell surface markers. Histogram plots represent staining of different cell surface molecules (unfilled) and isotype controls (filled). A representative experiment of several independent experiments is shown.

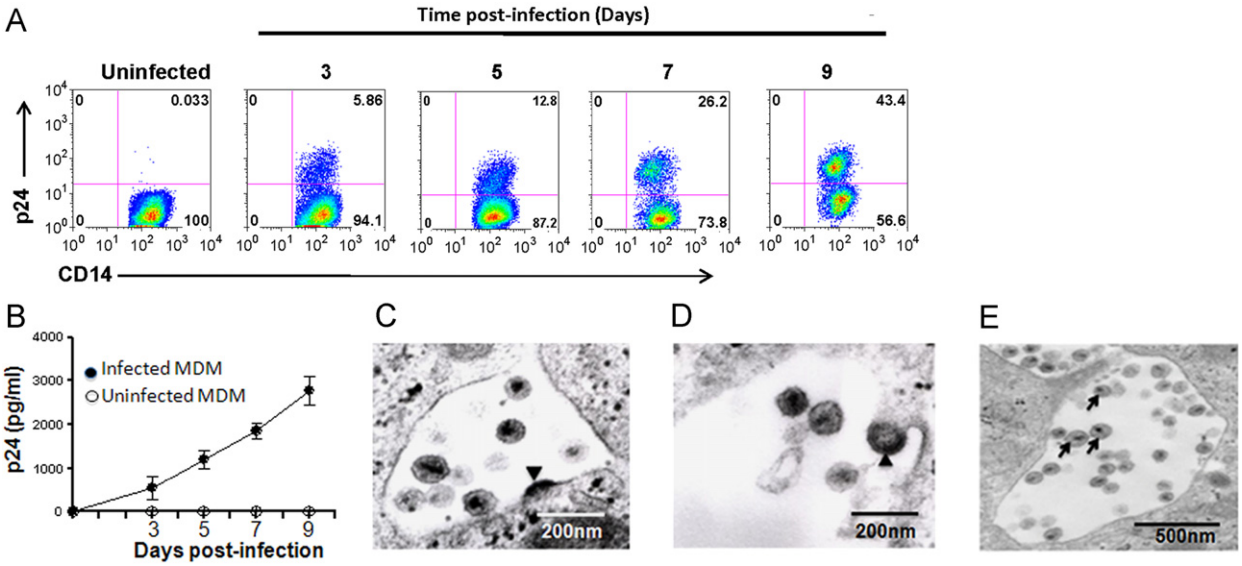

Fig. 2. HIV-1 infection of MDM. MDM were infected *in vitro* with purified US-1 virus (8 ng of p24) on day 7 of culture as described in the Materials and methods section. Cells and culture supernatants were harvested at the indicated time-points. Cells were stained and analyzed for the presence of intracellular p24 by flow cytometry. (A) Panels show dot plots of uninfected and US-1-infected MDM cultures at 5, 7, and 9 day post-infection. (B) The supernatants from these cells (infected MDM represented by closed circles and uninfected MDM represented by open circles) were collected at the indicated time-points and analyzed in triplicate for p24 levels by ELISA. (C)–(E) electron micrographs of MDM infected with US-1 at day 10 post-infection (C and D). HIV-1 can be seen budding (indicated by arrowheads) on the membrane of a vacuole (C) with budding nearing completion in (D). Bar=200 nm, respectively. (E) Arrows denote numerous US-1 virions inside a vacuole (Bar=500 nm). A representative of two separate experiments is shown.

Table 1
Binding specificities and endotoxin concentration for murine and human monoclonal antibodies. The endotoxin concentration in the murine and human mAb preparations was measured by using a commercially available limulus amoebocyte lysate (LAL) kinetic chromogenic assay kit (Kinetic-QCL) and the endotoxin units (EU) were calculated. The lipid and protein binding specificities were previously determined.

	Lipid and protein binding specificities*									Endotoxin concentration	
mAb	PIP	PIP2	PI	Cholesterol	GalCer	Lipid A	MPER	gp41	gp120	pg/10 μg	EU/10 μg
WR321 (IgG)	+	+	−	−	−	−	−	−	−	0.012	0.166
CL1 (IgG)	+	+	+	+	−	−	−	−	−	0.005	0.069
2F5 (IgG)	−	−	−	−	−	−	+	+	−	0.005	0.068

+: binding; −: no binding.

* Binding as previously determined (Alving et al., 1987; Matyas et al., 2009b, 2010).

used as positive controls. MDM infected with virus in the absence of preincubation with mAbs served as the infection control.

At day 5 post-infection, the percentage (mean ± S.D.) of MDM infected in the absence of mAbs (Media, Infection control) was 15.17 ± 3.26% (Fig. 3A, panel 2). When compared to the infection control, preincubation of MDM with 2F5 resulted in complete neutralization of intracellular US-1 virus infection (Fig. 3A, panel 3), whereas preincubation with CL1 neutralized the infection by 61.31 ± 3.89% (Fig. 3A, panel 4). Based on the measurement of intracellular and extracellular p24, there was a concomitant and significant decrease in the amounts of p24 in infected MDM preincubated with mAbs 2F5 and CL1 compared to the infection control indicating neutralization of the virus (Fig. 3B). In contrast, virus infection was not neutralized when MDM were preincubated with WR321 based both on the % of intracellular p24 (Fig. 3A, panel 5) and on the amount of intracellular and extracellular p24 compared to cells incubated with the virus alone (Fig. 3B).

In the virus format (Fig. 4), purified US-1 virus was preincubated separately with each of the mAbs. MDM infected with virus alone, served as the infection control (Media, Fig. 4A, panel 2). 2F5 and CL1 neutralized HIV-1 infection by 100% and 80.92 ± 4.12%, respectively ($p < 0.0001$) based on intracellular p24 (Fig.4A, panels 3 and 4) and measurement of both intracellular and extracellular p24 (Fig. 4B); whereas WR321 neutralized US-1 infection by 58.37 ± 3.54% based on intracellular p24 as measured by flow cytometry (Fig. 4A, panel 5) and ELISA (Fig. 4B). However, based on the amount of extracellular p24 (Fig. 4B), WR321 significantly neutralized the virus to 87% ($p=0.005$).

Binding of WR321 to HIV-1

The results of the virus neutralization assay format (Fig. 4) suggested that WR321 neutralized HIV-1 by binding to the virus. To conclusively demonstrate this, we utilized electron microscopy to visualize the binding of WR321 to US-1. Binding of WR321 to US-1 (Fig. 5A) was confirmed by the binding of the anti-IgG labeled-gold particles while the negative control IgG mAb, TFTB did not exhibit binding of the gold particles to US-1 (Fig. 5B).

The effect of donor variability and HIV-1 subtypes on the neutralizing effects of WR321

To further demonstrate that neutralization of HIV-1 by WR321 was not restricted to US-1 or to a particular donor, MDM

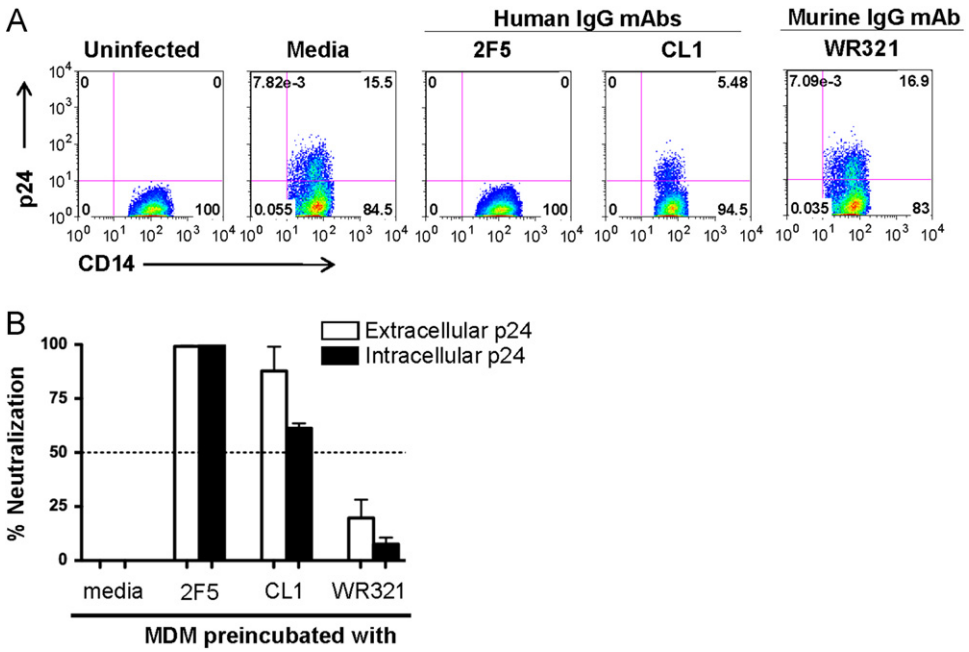

Fig. 3. Effect of preincubation of MDM with murine mAb WR321 on US-1 infection. MDM were preincubated with human IgG mAbs (2F5, CL1; panels 3 and 4) or murine IgG mAb (WR321, panel 5) for 1 h at 37 °C/5% CO_2. All mAbs were used at a final concentration of 10 μg/ml. Cells were washed and infected in triplicate with purified US-1 (8 ng). Cells preincubated with media (panel 1) and infected with US-1 served as the infection control (panel 2). Cells and culture supernatants were harvested on day 5 post-infection. Intracellular and extracellular p24 in MDM and culture supernatants were evaluated by flow cytometry and ELISA, respectively. (A) Dot plots show the percentages of MDM positive for intracellular p24. A representative of triplicate infections is shown. (B) Bar graph represents the % neutralization (mean ± S.D.) of HIV-1 infection by the different mAbs compared to the infection control based on the measurement of extracellular (open bars) and intracellular p24 (filled bars). Each of the samples from triplicate cultures was analyzed in duplicate. A representative of three independent experiments is shown.

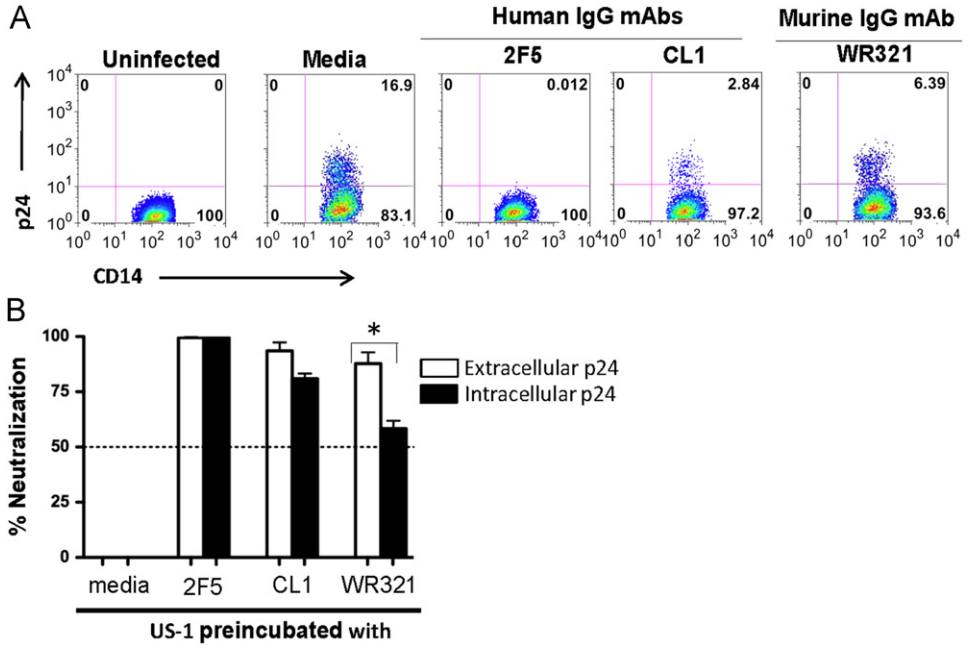

Fig. 4. Effect of preincubation of US-1 with murine mAb WR321. MDM were infected in triplicate with purified US-1 (8 ng of p24) that was previously preincubated with 10 μg/ml of human IgG mAb (2F5, CL1) or murine IgG mAb (WR321) for 1 h at 37 °C/5% CO_2. MDM infected with US-1 in the absence of mAbs served as the infection control. Cells and culture supernatants were harvested on day 5 post-infection. Intracellular and extracellular p24 in MDM and culture supernatants were evaluated by flow cytometry and ELISA, respectively. (A) Dot plots show the percentages of MDM positive for intracellular p24. A representative of triplicate infections is shown. (B) Bar graph represents the % neutralization (mean ± S.D) of HIV-1 infection by the different mAbs compared to the infection control based on the measurement of extracellular (open bars) and intracellular (filled bars) p24. Each of the samples from triplicate cultures was analyzed in duplicate. A representative of three independent experiments is shown. Neutralization that is significant based on extracellular p24 measurement compared to intracellular p24 measurement is represented by an * ($p=0.001$).

differentiated from three healthy HIV-1 seronegative donors (#032, #124, #202) and two additional primary viruses, BaL (clade B) and MO66 (clade A/E) were utilized along with 2F5 and CL1 as the control mAbs (Table 2; Fig. 6). All infections were done in triplicate and the neutralizing effect of the three mAbs on the three virus subtypes was determined on day 5 post-infection. MDM infected with the respective purified virus subtypes in the absence of mAbs served as the infection control.

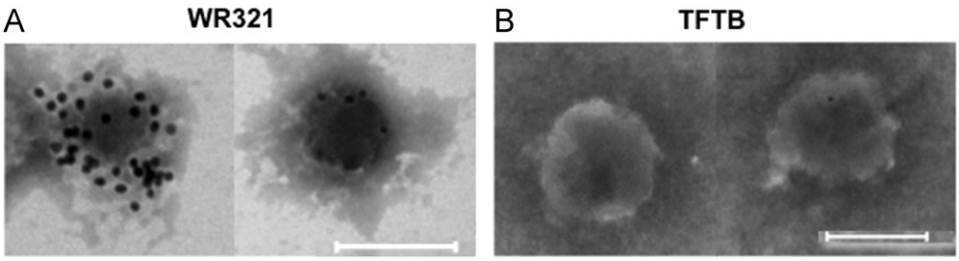

Fig. 5. Visualization of binding of WR321 to HIV-1. Monoclonal antibodies WR321 (panel A) and TFTB (panel B) were preincubated with purified US-1 virus for 16 h at 4 °C in duplicate. The virus-mAb mixtures were placed on formar-coated copper grids followed by gold labeled-goat anti-mouse IgG antibody (10 nm gold particles) as described in the Materials and methods section. The grids were washed, stained with 1% phosphotungstic acid, and examined under a Phillips CM100 Transmission Electron Microscope. Numerous gold particles can be seen attached to US-1 preincubated with WR321 (panel A) compared to US-1 preincubated with TFTB-1 (control antibody, panel B). Two independent experiments were carried out and in each case approximately 15 fields were examined. A representative field is shown (Bar=100 nm).

Table 2
Infectivity of MDM from different donors with different HIV-1 subtypes. MDM were prepared from three seronegative donors and infected *in vitro* in triplicate with HIV-1 subtypes (US-1, BaL, and MO66). Cells were harvested on day 5 post-infection and evaluated for the presence of intracellular p24 by flow cytometry. These experiments were performed twice. Data are expressed as % of p24 positive cells (Mean ± S.D.).

Percentage of p24-positive MDM (Mean ± S.D.)			
Donor	US-1 (Clade B)	BaL (Clade B)	MO66 (Clade CRF01_AE)
#032	23.13 ± 2.98	43.34 ± 4.87	12.51 ± 1.51
#124	30.79 ± 2.88	31.79 ± 4.02	22.34 ± 3.48
#202	5.66 ± 0.71	28.55 ± 2.42	12.57 ± 2.41

As shown in Table 2, MDM from the three donors showed different degrees of susceptibility to infection with the different HIV-1 subtypes. The variability in HIV-1 susceptibility among the three different donors is not surprising since the capacity of MDM to sustain viral replication varies between individuals probably due to certain host or innate factors that could limit several steps of the viral life-cycle from viral entry to virus release (Eisert et al., 2001).

WR321 did not neutralize any of the three viruses tested in any of the three donor MDM tested (Fig. 6A–C) when the mAb was preincubated with MDM (cell format). Neutralization as determined by the amount of extracellular or intracellular p24 in all cases based on the infection control was well below the 50% cut off. In contrast, 100% neutralization was consistently obtained with the mAb 2F5 for all three viruses in all three donor MDM, while 70% to 80% neutralization was obtained with the mAb CL1 when it was used either in the cell (Fig. 6A–C) or the virus format (Fig. 6D–F).

Pre-incubation of WR321 with all three viruses resulted in 60% to 80% neutralization with MDM from all three donors with a significantly higher degree of neutralization of all three viruses based on the measurement of extracellular p24 compared to the measurement of intracellular p24 (Fig. 6D–F). There were no significant differences in neutralization of any of the viruses tested with the control mAbs 2F5 and CL1 based on the measurement of intracellular or extracellular p24.

Internalization of WR321 and chemokine production

To conclusively prove that the virus-WR321 complex was internalized, MDM were incubated with media, WR321, US-1, or US-1 pre-incubated with WR321 as described in Materials and methods Section and then analyzed for the presence of intracellular p24 and intracellular WR321 by flow cytometry. As shown in the histograms (Fig. 7A) and Table 3, a shift in the mean fluorescent intensity (MFI) of the cells was observed only when MDM were treated with WR321 preincubated with US-1. The MFI of cells that showed WR321 staining was 3-fold higher when MDM were cultured with HIV-1 preincubated with the mAb WR321 compared to MDM exposed to HIV-1 alone, WR321 alone, or to fluorescently labeled secondary antibody alone that was added after the cells were fixed and permeabilized. The level of infection of the cells under these same conditions was measured by staining for intracellular p24. The amount of p24 present in the cells at 36 h was low; nonetheless a slight shift in the MFI for p24 staining was only seen in cells incubated with virus alone or with virus preincubated with WR321 (data not shown). These results indicate that WR321 is internalized only when it is bound to the virus and not in the absence of the virus.

Supernatants from MDM cultured under the four experimental conditions described above were assayed for the presence of chemokines MIP-1α (Fig. 7B) and MIP-1β (Fig. 7C). MDM cultured with media alone produced little or no chemokines. WR321 had extremely low levels of endotoxin (Table 1) and incubation of MDM with the mAb alone induced very low amounts of MIP-1α and MIP-1β. Slightly higher amounts of both chemokines were induced with virus alone. As predicted, chemokine production was significantly higher in the MDM cultures containing virus preincubated with WR321. A similar pattern of chemokine production was seen in culture supernatants collected after 5 days of culture under the four experimental conditions described above (data not shown).

Discussion

The primary targets of HIV-1 are $CD4^+$ T cells and macrophages. In addition to their highly phagocytic nature, macrophages are professional antigen presenting cells, one of the first responders at the site of an infection, secrete a number of different cytokines and chemokines, and serve as a bridge between the innate and adaptive immune systems. Because of their long half-life of several months and their relative resistance to the cytopathic effects, tissue macrophages can harbor HIV-1 for extensive periods of time producing large amounts of virions (Carter and Ehrlich, 2008).

In primary CD4+ T cells, cellular entry of HIV-1 requires a fusion event between the cellular membrane and the viral lipid envelope while HIV-1 assembly and release occurs at the plasma membrane involving lipid rafts (Nguyen and Hildreth, 2000). In contrast, the mode of entry and exit of HIV-1 in primary human macrophages is not very clear. In these cells, fusion and endocytosis, macropinocytosis, and involvement of Fc receptors (Miyauchi et al., 2009; Marechal et al., 2001) have all been

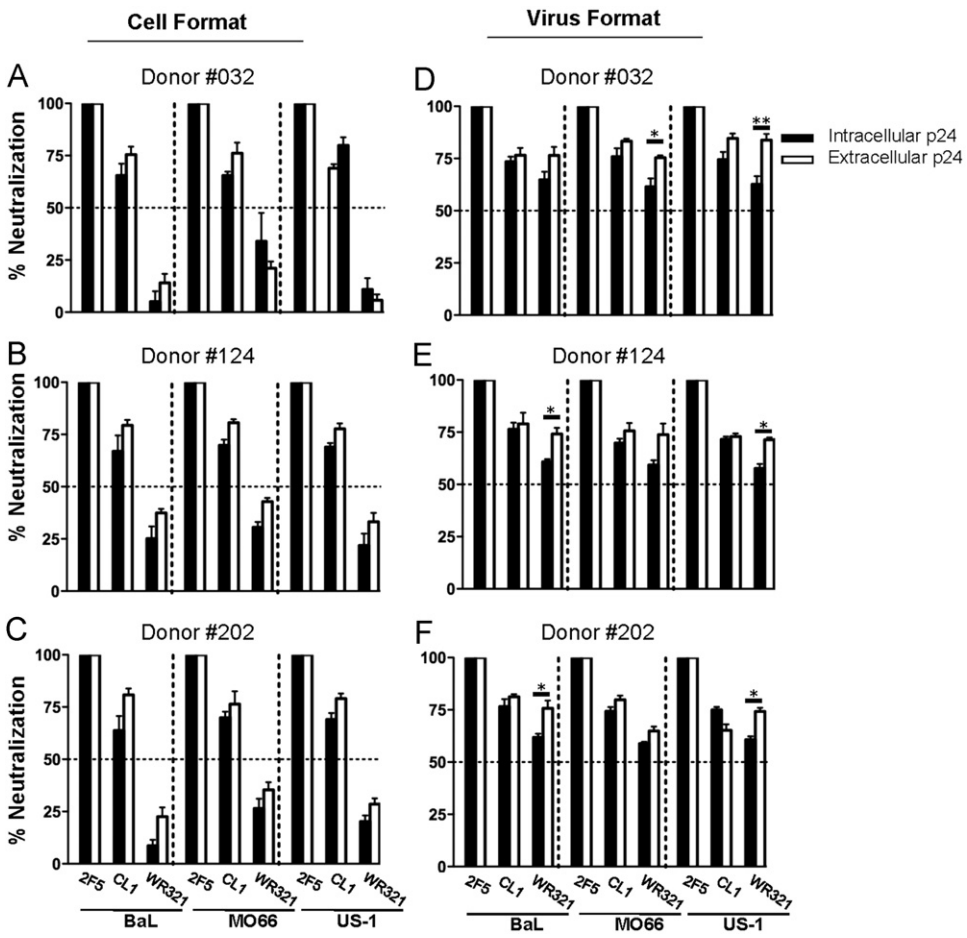

Fig. 6. Neutralization of HIV-1 infection by mAbs. The ability of murine mAb WR321 to neutralize HIV-1 infection (US-1, BaL, MO66) was assayed with MDM prepared from three seronegative donors (#032, #124, #202) under the two experimental conditions (cell format and virus format). In the cell format (panels (A)–(C)), triplicate wells of MDM were preincubated with mAb for 1 h washed, and subsequently infected with three HIV-1 subtypes. In the virus format (panels (D)–(F)), the three HIV-1 subtypes were preincubated with mAb for 1 h and subsequently added to triplicate wells of MDM. 2F5 and CL1 were used as control mAbs. MDM infected with the HIV-1 subtypes in the absence of mAb served as the infection control. HIV-1 subtypes were used at 8 ng in all the infections. All mAbs were used at a final concentration of 10 μg/ml. Cells were harvested on day 5 post-infection and analyzed for the presence of intracellular p24 by flow cytometry. (A)–(C) Bar graphs show the % neutralization of HIV-1 infection of MDM in the cell format. (D)–(F) Bar graphs show the % neutralization of HIV-1 infection of MDM in the virus format. Values shown are the mean ± standard deviation of triplicate samples. Percent neutralization (mean ± S.D) of HIV-1 infection by the different mAbs compared to the infection control is based on the measurement of extracellular and intracellular p24. A representative experiment of three independent experiments is shown. Significant differences in neutralization of intracellular *versus* extracellular p24 by WR321 mAb are represented by an ∗ (∗, $p=0.03$; ∗∗, $p=0.007$).

documented as routes of entry, and late endosomes as well as the plasma membrane have been demonstrated as the sites of HIV-1 assembly and budding (Deneka et al., 2007; Welsch et al., 2007). However, it is clear that both cholesterol and lipid rafts are important for virus entry and for budding irrespective of the cell type (Carter et al., 2009). Retroviruses, including HIV, acquire their lipid coats by budding through host plasma membranes (Brugger et al., 2006; Nguyen and Hildreth, 2000). A comprehensive mass spectrometry analysis of the lipid content of HIV-1 demonstrated that it was enriched in cholesterol, ceramide, ganglioside GM3, PIP, and PIP2 (Chan et al., 2008). Clearly, lipids are important in HIV-1 infection and generation of antibodies against lipids may be important for neutralization of HIV-1 infection in susceptible target cells.

In the current study, we investigated the inhibition of HIV-1 infection by WR321 and the mechanism of neutralization. For this purpose, we set up a human MDM system as target cells for HIV-1 infection. Unlike, the macrophage single cycle infection utilized by Holl et al., (2004), our MDM system consisted of multiple rounds of infection. HIV-1 was neutralized only when WR321 was preincubated with the virus and not when the mAb was pre-incubated with MDM. Therefore, it is reasonable to assume that this outcome was due to WR321 binding to the virions. WR321 binds exclusively to PIP and PIP2. Phosphoinositides that are recognized by WR321 do not exist on the external surface of cells, but are present in relatively high concentrations on the inner leaflet of the plasma membrane lipid bilayer (Di Paolo and De Camilli, 2006) as well as on the surface of virions and largely acquired during viral budding. Binding of WR321 to HIV-1 was conclusively demonstrated by electron microscopy. In our study, we have used a defined MDM system which is not a mixture of many different cell types as is the case with PBMCs and showed that WR321 neutralized MDM infection by binding to the virions. Furthermore, there was a significant increase in both MIP-1α and MIP-1β production only when MDM were cultured with WR321 that had been pre-incubated with HIV-1 compared to MDM cultured just with WR321 or HIV-1.

It has been demonstrated that interference in the interaction between HIV-1 Gag protein and PIP2 leads to defective non-infectious particles (Chan et al., 2008; Ono et al., 2004). Our data demonstrate that WR321 mediates its effect only when it is bound to the virus and internalized by MDM. In addition to triggering the release of chemokines, we hypothesize that WR321 because of its exclusive specificity to phosphoinositides binds to

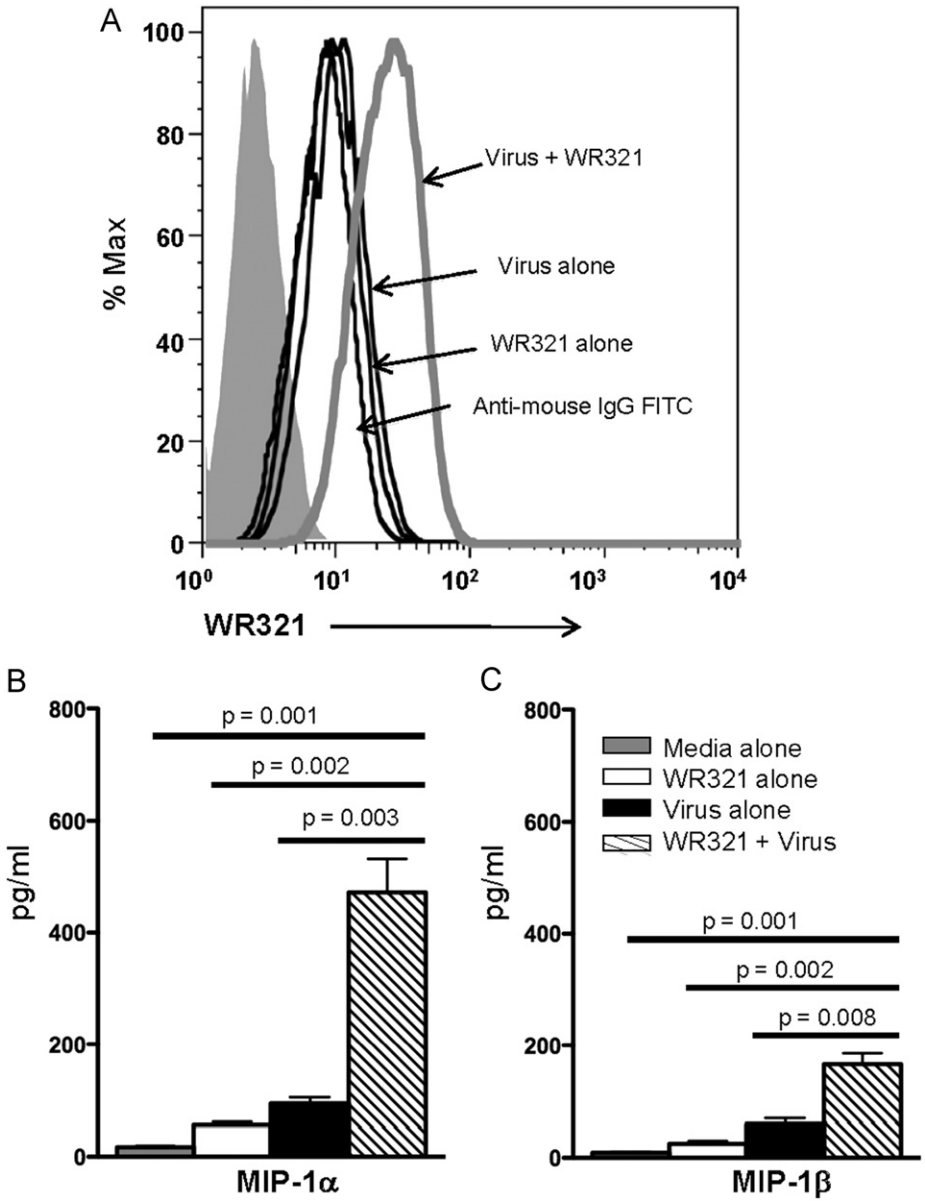

Fig. 7. Internalization of WR321 and induction of β-chemokines. MDM were incubated with media, WR321, US-1, or WR321 preincubated with US-1 as described in Materials and methods section. Cells and culture supernatants were harvested. Cells were fixed, permeabilized, and stained with FITC-conjugated anti-mouse mAb. A separate aliquot of fixed and permeabilized MDM were stained with FITC-conjugated anti-mouse mAb as a control. The gray histogram represents unstained cells. Histograms show the detection of WR321 staining in the MDM preparations as visualized by flow cytometry (A). Culture supernatants were analyzed for MIP-1α and MIP-1β by ELISA. Bar graphs show the concentration of MIP-1α (B) and MIP-1β (C). The mean ± S.D. of triplicate samples is shown. Statistical significance for amount of β-chemokine production was assessed by Mann–Whitney *t*-test and the *p* values are indicated in the figure.

Table 3
Mean fluorescence intensity (MFI) of WR321 staining. MDM were incubated with media alone, WR321, US-1 alone, or WR321 preincubated with US-1 as described in the Materials and methods section. Cells were harvested, fixed, permeabilized, and stained with FITC-conjugated anti-mouse mAb to visualize WR321 by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) of WR321 staining in the MDM preparations under the various conditions (Fig. 7).

Condition	MFI
Media alone	2.6
WR321 alone	8.3
Virus alone	8.5
FITC conjugated anti-mouse alone	7.9
WR321 preincubated with virus	25.7

PIP and prevents either the proper assembly or the budding of the virions from MDM. This hypothesis is supported by the observed differences seen in the neutralization of HIV-1 by WR321 based on the measurement of intracellular and extracellular p24. A significantly greater degree of neutralization was observed based on the amount of extracellular p24 (87% neutralization) measured compared to intracellular p24 (58% neutralization, Fig. 4B). These results would suggest that HIV-1 virions are probably contained within the cell and are prevented from exiting the cell thereby leading to lower amounts of p24 in the culture supernatant compared to infection control resulting in the observed greater neutralization. Such a difference in inhibition of HIV-1 infection based on the intracellular and extracellular p24 measurement

was not observed with the mAb 2F5 since its mechanism of action is at the entry stage. To the best of our knowledge, WR321 is unique in this aspect and probably the first anti-phosphoinositide mAb to exert its effects from within the cell by preventing the release of virions and also triggering the release of chemokines. The chemokines would then bind to the chemokine receptor CCR5 and inhibit the binding of virions.

A major goal in the area of HIV-1 research is to induce the production of broadly cross-neutralizing antibodies. However, immunization strategies aimed at generating these types of antibodies with broad neutralizing capabilities have so far been futile largely due to the lack of understanding of the mechanism of production of these antibodies (Montero et al., 2008; Phogat et al., 2008). Furthermore, it is important to realize that *in vivo* the virus encounters several different cell types and the methods of viral entry are varied. Thus, it is important to not only develop antibodies that are not restricted in their inhibition to one cell type and would work with all hematopoietic cells, but utilize several different assays systems to measure neutralization. WR321 not only neutralizes HIV-1 in a macrophage system by preventing the proper assembly and release of the virions and by the induction of chemokines, but also neutralizes HIV-1 in a PBMC neutralization assay as was previously shown (Matyas et al., 2010). Therefore, several different assay systems should be utilized to screen antibodies generated by potential candidate vaccine formulations before drawing major conclusions about their feasibility in clinical trials.

Materials and methods

Antibodies and reagents

Monoclonal antibodies anti-CD3-FITC, CD4-PE, HLA-A, B, C-FITC, CD4-FITC, CD40-FITC, CD11c-PE, CD11b-PE, CD195-PE, CD80-PE, CD64-PE, CD184-PE, CD14-PerCP, CD20-APC, CD56-APC, HLA-DR-APC, CD86-APC, and CD1a-APC, were obtained from BD Pharmingen. Anti-p24-FITC and anti-p24-PE were purchased from Beckman Coulter. WR321 (murine IgG) was generated in our laboratory (Matyas et al., 2010, 2009b; Wassef et al., 1984). CL1, a human mAb derived from a patient with systemic lupus erythematosus, with specificity for cardiolipin, phosphoinositides, cholesterol, and sulfatide (Moody et al., 2008; Matyas et al., 2009a, 2010), was provided by Dr. Barton Haynes (Duke University Medical Center, Durham, NC). Anti-gp41 MPER mAb 2F5 was purchased from Polymun Scientific Immunbiologische Forschung GmbH. TFTB, a mouse IgG mAb to the ricin B chain was purified as previously described (Matyas et al., 2010) and was used as a negative control antibody. Polybrene was purchased from Sigma-Aldrich and formar-coated 300 mesh copper grids were obtained from Electron Microscopy Sciences.

Endotoxin detection

Endotoxin levels in WR321, 2F5, and CL1 were measured by using a commercially available limulus amoebocyte lysate (LAL) kinetic chromogenic assay kit (Kinetic-QCL, Lonza). The assay was carried out according to the manufacturer's instructions. The lipid and protein binding specificities of the murine and human mAbs and their endotoxin levels are shown in Table 1.

Virus purification

HIV-1 stocks of primary isolates of US-1 and BaL (clade B) and MO66 (clade CRF01_AE) grown in PBMCs were provided by Dr. Victoria Polonis (USMHRP). The supernatant containing the virus was collected either on day 4 or on day 5, clarified by centrifugation at $311 \times g$ for 10 min, and passed through a 0.22 μm filter. The culture supernatant was subjected to ultracentrifugation at $100{,}000 \times g$ for 90 min at 4 °C and the virus pellet was resuspended in 400 μl of PBS. The virus was mixed with protein A/G beads that had been pre-coated with anti-acetylcholinesterase antibody (10 μg/20 μl of beads) and rotated for 60 min at 4 °C. The mixture was centrifuged in a microcentrifuge at $16{,}110 \times g$ for 1 min to pellet the beads. The supernatant was placed in a clean tube and subjected to a second round of immunodepletion to remove any contaminating exosomes present after the first round of depletion (Cantin et al., 2008). Anti-CD45 antibody cocktail was then added to the supernatant for 15 min at RT followed by the addition of magnetic nanoparticles (CD45 depletion kit, StemCell Technologies, Vancouver, Canada) for 10 min at RT to remove microsomes (Trubey et al., 2003). Samples were placed on a magnet for 3–5 min and the supernatant was removed. Virus samples were stored in liquid nitrogen until use in aliquots containing 15% FBS that had been subjected to ultracentrifugation to remove bovine exosomes. Infectivity and p24 concentration were determined before and after purification to ensure that infectivity was not lost during the purification procedure.

Preparation of human monocyte-derived macrophages (MDM)

For the generation of MDM, PBMCs from healthy volunteers were collected under an internal review board (IRB)-approved protocol, RV229/WRAIR number 1386. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of HIV-1 seronegative donors using the cold agglutination procedure (Mentzer et al., 1986). Briefly, 50×10^6 PBMCs were resuspended in 1.2 ml cold monocyte media (RPMI supplemented with 10% heat-inactivated FBS, 1% L-glutamine plus 1% penicillin/streptomycin) in a 15 ml centrifuge tube and slowly rotated horizontally for 45 min at 4 °C. The tube was placed vertically on ice for 30 min and then 1.5 ml of cold FBS was slowly dispensed under the cell pellet. Following aspiration of the media and FBS, the cell pellet was resuspended in 10 ml monocyte media and spun at $311 \times g$ for 10 min at 4 °C. The cell pellet was resuspended in macrophage media [monocyte media supplemented with 50 ng/ml macrophage colony stimulating factor (M-CSF)]. Cell viability as determined by trypan blue exclusion was $>99\%$. The purity of the monocytes ($>90\%$) was determined by staining with anti-CD14 and anti-CD11b mAbs and analyzed by flow cytometry (FACSCalibur, BD Biosciences). Monocytes were cultured in 24-well plates at a concentration of 1×10^6 cells/well. The culture media was changed every two days and MDM were used at day 7 post-culture.

Flow cytometry

Briefly, monocytes or MDM (1×10^6 cells/ml) were resuspended in cold FACS buffer (PBS-containing 0.5% BSA) and incubated with 5 to 10 μg of the relevant mAbs directed against cell surface molecules for 20 min at 4 °C. Cells were washed in FACS buffer and fixed in PBS-containing 2% paraformaldehyde. Flow cytometry was performed on a FACSCalibur. Data was analyzed using FlowJo 8.8.6 software (TreeStar Inc.).

In order to determine if WR321 binds to MDM, cells ($0.5–1 \times 10^6$ cells/ml) were washed in FACS buffer and resuspended in 100 μl blocking buffer (PBS-containing 20% normal goat serum) for 90 min at 4 °C. For evaluation of extracellular binding, WR321 mAb (10–50 μg/ml) was then added to the cells in the presence of anti-CD14 mAb and the cells were incubated for an additional 60 min at 4 °C. Following two washes in FACS

buffer, the cells were resuspended in 100 μl FACS buffer containing 5 μg of goat anti-mouse FITC secondary Ab. Cells were then incubated for 45 min at 4 °C. Cells were washed in FACS buffer and fixed in PBS-containing 2% paraformaldehyde. Flow cytometry and data analyses were performed as mentioned above.

Electron microscopy

MDM were infected with purified US-1 virus (see above). Cells were collected on day 10 post-infection, washed, and then incubated with fixative (PBS-containing 4% formaldehyde and 1% glutaraldehyde) for 1 h at RT followed by overnight incubation at 4 °C. Following washing with phosphate buffer, the cells were post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol and embedded in Embed 812 resin (Electron Microscopy Sciences). Thin sections (80–100 nm) were cut, stained with lead citrate and uranyl acetate and examined using a LEO 912 Electron microscope (Carl Zeiss SMT).

In order to determine the binding of WR321 to HIV-1, the mAb was preincubated with purified US-1 virus at a final concentration of 10 μg/ml for 16 h at 4 °C. TFTB (10 μg/ml) served as a negative control. The virus-mAb mixture was placed on formar-coated 300-mesh copper grids that were previously coated with polybrene (5 μg/ml). Following incubation at RT for 5 min, the grids were blocked, with 4% goat sera in PBS for 10 min. Goat anti-mouse IgG antibody labeled with 10 nm gold particles (Electron Microscopy Sciences) was added and the grids were incubated for 60 min at RT. The grids were gently washed in PBS and incubated in fixative (PBS-containing 4% formaldehyde and 1% glutaraldehyde) for 20 min at RT. The grids were washed, stained with 1% phosphotungstic acid, and examined under a Phillips CM100 Transmission Electron Microscope (FEI).

HIV-1 infection of MDM and measurement of intracellular p24

All incubations containing MDM in this experiment and subsequent experiments were carried out at 37 °C/5%CO_2. MDM (1×10^6) grown in 24-well plates were incubated with 500 μl of fresh infection media (macrophage media-containing 2 μg/ml polybrene) for 1 h. The media was aspirated, 300 μl of infection media containing purified US-1, BaL, or M066 virus (8 ng of p24) was added to the wells. The plate was centrifuged at $863 \times g$ for 1 h at 37 °C to allow the virus to gain better access to the cells, and then incubated for 4 h. The virus suspension was then aspirated, cells were extensively rinsed with monocyte media containing 2 μg/ml polybrene to remove unbound virus, and the plate was incubated in 1 ml of fresh infection media for 5 days.

For detection of HIV-1-infected macrophages ($CD14^+$ $p24^+$ cells), MDM were harvested, washed, and incubated in FACS buffer containing 5–10 μg of anti-CD14 mAb for 20 min at 4 °C. Following washing in FACS buffer, the cells were fixed and permeabilized according to the manufacturer's instructions (Caltag), and then stained with anti-p24-FITC (Beckman Coulter) or with anti-p24-PE for 15 min at RT. Uninfected cells stained with anti-p24-FITC or anti-p24-PE as well as infected cells stained with FITC- or PE-conjugated IgG1 served as the controls. Cells were washed in FACS buffer, resuspended in PBS and evaluated by flow cytometry using a FACSCalibur. Data was analyzed using FlowJo 8.8.6 software and the % of cells positive for intracellular p24 was calculated.

Measurement of extracellular p24

Supernatants harvested from HIV-1 infected MDM cultures were assayed in triplicate for the presence of p24, by an ELISA according to the manufacturer's instructions using a HIV-1 p24 antigen capture assay kit (Advanced BioScience Laboratories).

Antibody neutralization assays

Two separate formats, cell format (A) and virus format (B) were utilized. In the cell format, WR321 was preincubated with MDM. In the virus format, WR321 was preincubated with the virus.

A) Cell format: To determine whether WR321 could bind to MDM and neutralize HIV-1 infection, MDM (1×10^6 cells/well in 24-well plates) were preincubated with the mAb at for 1 h. The final mAb concentration in the mixture in each case was 10 μg/ml. Pooled human sera from HIV-1 negative individuals and mAb 2F5 served as the negative and positive controls, respectively. After aspiration of the unbound mAb, the cells were rinsed and 300 μl of infection media containing purified US-1, BaL, or MO66 was then added to the wells, and the plate was centrifuged at $863 \times g$ for 1 h at 37 °C. The plate was then incubated for 4 h. Following aspiration of the virus suspension, the cells were extensively rinsed, 1 ml of fresh infection media was added, and the plate was incubated for 5 days. MDM and culture supernatants were harvested and analyzed respectively for the presence of intracellular and extracellular p24.
B) Virus format: To determine whether WR321 could bind to different virus isolates and neutralize infection of MDM, the mAb was preincubated with purified US-1, BaL, or MO66 (8 ng of p24). The final mAb concentration in the mixture in each case was 10 μg/ml. The positive and negative controls were the same as that used for the cell format. Following incubation for 1 h, the virus-mAb mixture was spun in a microfuge at $16{,}110 \times g$ for 30 min. The supernatants were aspirated to remove the unbound mAbs. The individual virus pellets were resuspended in 300 μl of infection media and added to the respective wells of a 24-well plate containing MDM (1×10^6/well). Following centrifugation at $863 \times g$ for 1 h at 37 °C, the plate was further incubated for 4 h. After aspiration of the virus suspension, cells were extensively rinsed and incubated in 1 ml of fresh infection media for 5 days. MDM and culture supernatants were harvested and analyzed respectively for the presence of intracellular and extracellular p24. It was determined in separate experiments that pelleted virus in the absence of mAbs exhibited the same level of infectivity as the unpelleted virus (data not shown). In both the cell and virus formats, the % of neutralization was calculated based on the amount of p24 in the presence and in the absence of the mAbs.

Internalization of WR321 and measurement of β-chemokines

WR321 (10 μg/ml) was preincubated with US-1 (8 ng of p24) for 1 h at 37 °C/5% CO_2 and the mixture added to wells of a 24-well plate containing MDM (1×10^6 cells/well). Media alone, media-containing WR321, or US-1 alone was added to corresponding wells of MDM. The plate was centrifuged at $863 \times g$ for 1 h at 37 °C and further incubated for 36 h after which the cells and culture supernatants were harvested. Cells were washed with FACS buffer and stained with CD14 mAb. Following washing, the cells were fixed and permeabilized (Caltag), according to the manufacturer's instructions. The cells were stained for 1 h at RT either with anti-p24-PE or with goat anti-mouse-FITC secondary mAb in permeabilization buffer to visualize for the presence of HIV-1 or WR321. The cells were washed in FACS buffer,

resuspended in PBS and evaluated by flow cytometry using a FACSCalibur.

Supernatants harvested from MDM cultures were assayed in triplicate for the presence of MIP-1α and MIP-1β by ELISA according to the manufacturer's instructions using the respective chemokine ELISA kits (R & D Systems).

Statistical analyses

Data are presented as the mean ± S.D., and the statistical differences among groups were analyzed by the Mann–Whitney *U* test using Graphpad Prism 5.0 software.

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